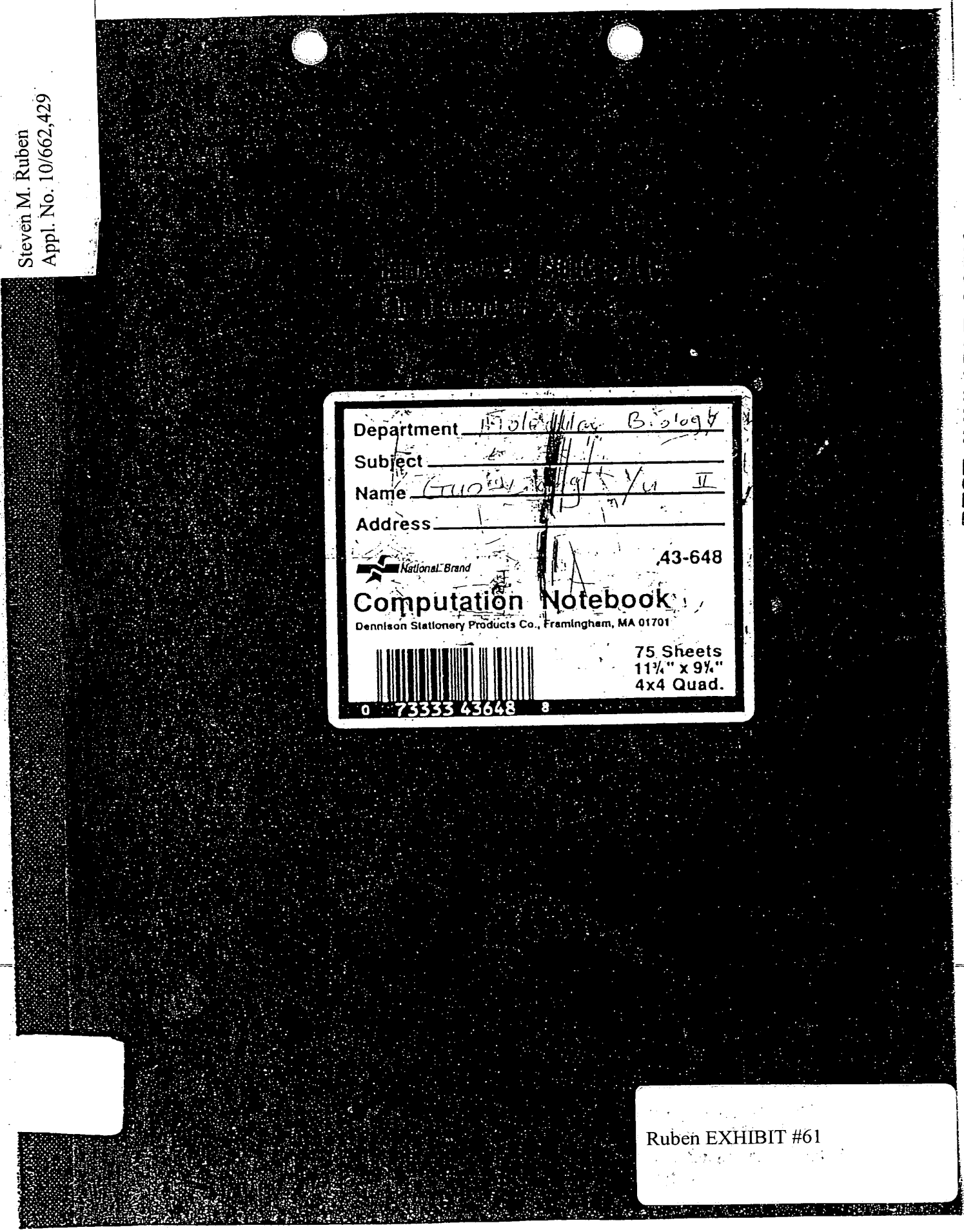
Steven M. Ruben
Appl. No. 10/662,429



BEST AVAILABLE COPY

Ruben EXHIBIT #61

Department Molecular Biology

Subject

Name Guo-Liang Yu II

Address

National Brand 43-648

Computation Notebook

Dennison Stationery Products Co., Framingham, MA 01701

0 73333 43648 8

75 Sheets
11¾" x 9¼"
4x4 Quad.

8/21/95 Two New TNF: HPDD012 TNF Epsilon
HETBT71 TNF delta
there are differences between the two clones, maybe splice variant

Oligo captive cloned full length Named HETBT71SQ7

design oligos for seq Rpo2 CTCCCAGCTTGGAAGACCAG
Rpo3 TTCTGCATGCCCACACCTCTC
Fpo6 GAACAGAAGCAAGATATCCG

design oligos for δ-specific Cintron J?

design oligos for construct into pQE70

TNFδ-SphI: CGCGCATGCAGGGGACAGGAGGCC
TNFδ-HindIII: CGCAAGCTTACAATCACAGTTTCACAAC

TNFδ-specific oligo is given to L. Xing for Northern

8/25/95 PCR to generate insert DNA for following constructs

1. TNF5 SphI + HindIII ⟹ pCET0
2. TNFe SphI + HindIII ⟹ pCET0
3. TNFr dig NcoI + HindIII ⟹ pCET60
4. TNFr FL BamHI + Bam H3' ⟹ N346
5. TNFr FL Bam H + Bam H3' ⟹ New C40 vector

10 λ 10X PCR buf BM
10 λ 2mM dNTP
oligos
0.5 μl PWO polymerase

100 μl

95C 2' 30[95C 1' 55C 1' 72C 1'] 75C 5 min

PCR ⟹ low melting gel (Nusieve)

± 4 PCR for C40 clone did not work

repeat PCR

SUPERVISOR- Patrick J. Allen
DATE- 08/31/95

9/4/95 PCR → product BamHI fragment for TNFr

& extract 2x

DNA ref

cells ext

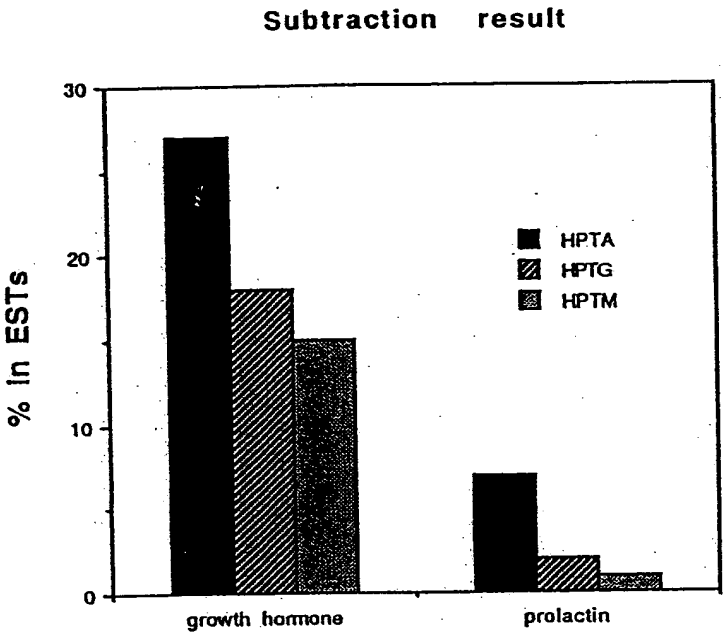
ethanol ppt

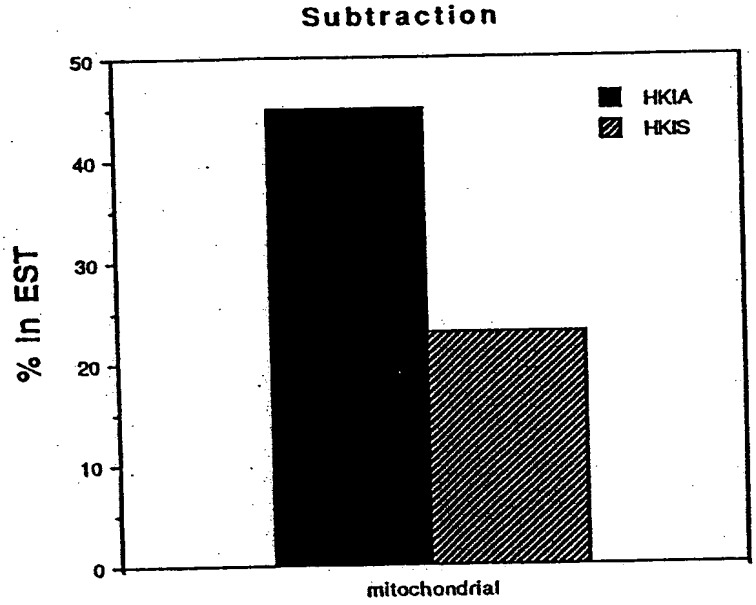
I cut — check 1Δ on gel

give to Lily for cloning into CHO vectors

9/6/95 Summary on subtraction using biotinylated drivers generated by PCR using biotin-dCTP

	Driver	PRL	G4	mito
HPTA	—	6.5%	27%	—
HPTG	G4 PL	1.6%	18%	—
HPTM	G4 PL	1.2%	15%	—
HSDB	—			34%
HSDS	mito			28%
HKIA	—			45%
HKIS	mito			23%


Subtraction result
30
20
10
0
% In ESTs
HPTA
HPTG
HPTM
growth hormone
prolactin


Subtraction
50
40
30
20
10
0
% In EST
HKIA
HKIS
mitochondrial

9/6/95 Modify the approach:

① using regulator drop to generate ss DNA

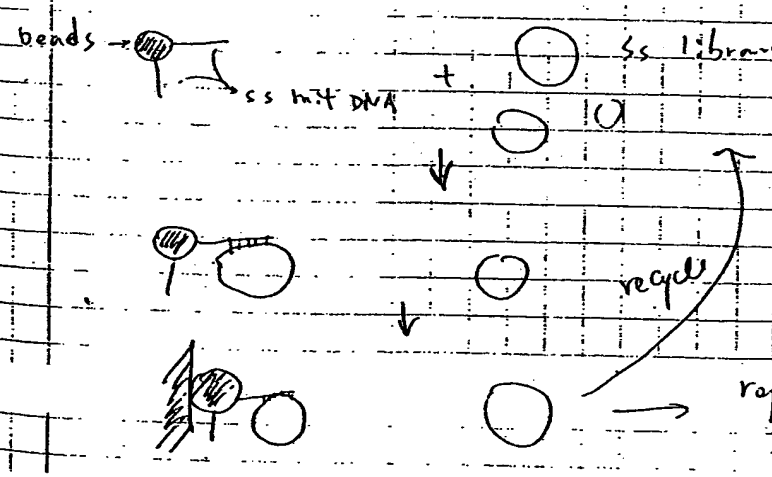
② photo biotin DNA

this is proven to be efficient

③ Try both Long hybridization at 42°C o/n and short hybridization at RT 1 hr

④ remove biotin by extract add one more step should no difference by using magnetic beads

9/15 Discussion w/ Fouad idea



NORTHERN BLOT DATA SHEET

OPERATOR: Lily Xing DATE 9/1/94

LANE #	NAME	RNA (µl/10µg)	Note
1	Brain	20 ug	
2	kidney	"	
3	small intestin	"	
4	testis	"	
5	pancreas	"	
6	prostate	"	
7	Heart	"	
8	Liver	"	
9	Lung	"	
10	Thymus	"	
11	spleen	"	
12	placenta	"	
13	Colon	"	
14	ovary	"	
15	Leukocyte	"	
16	muscle	"	
17			
18			
19			
20			

NOTE:

#3 gel

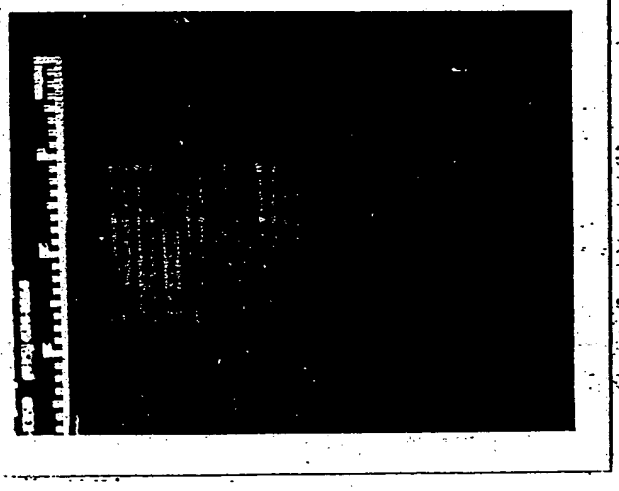
NORTHERN BLOT DATA SHEET

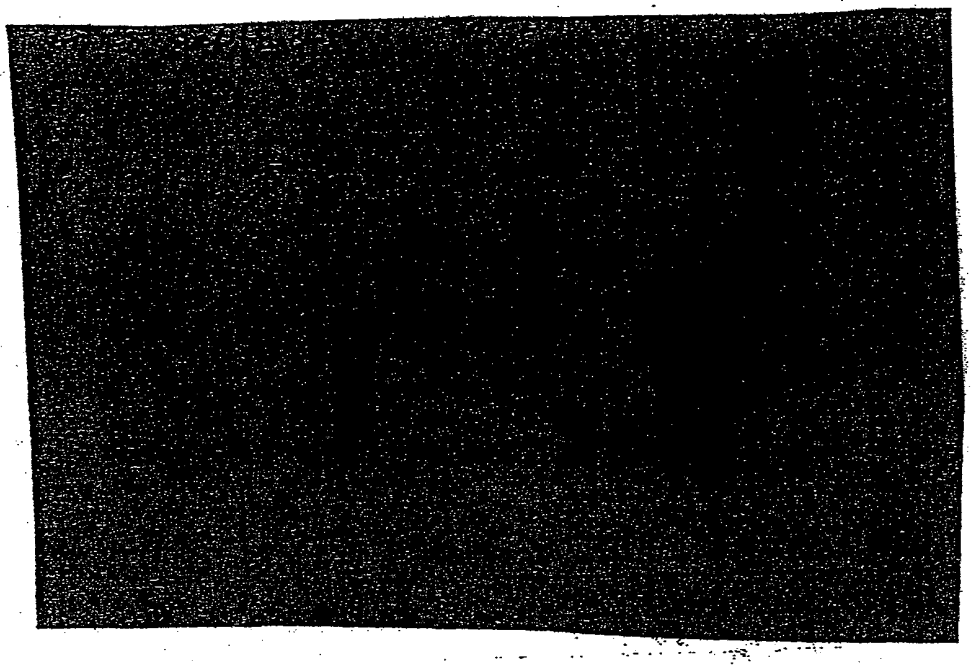
OPERATOR: Lily Xing DATE 9/1/94

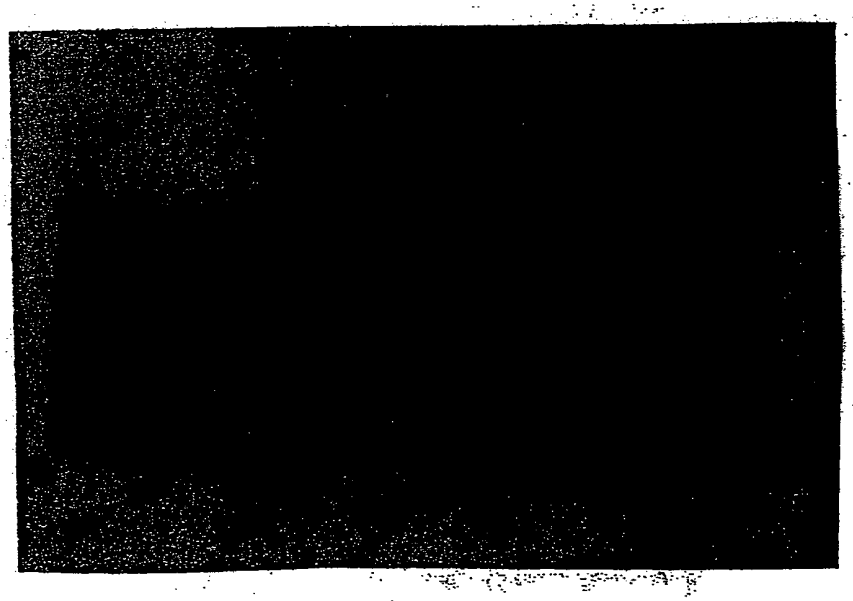
LANE #	NAME	RNA (µl/10µg)	Note
1	Brain	20 ug	
2	kidney	"	
3	Small intestin	"	
4	testis	"	
5	pancreas	"	
6	prostate	"	
7	Heart	"	
8	Liver	"	
9	Lung	"	
10	thymus	"	
11	spleen	"	
12	placenta	"	
13	Colon	"	
14	ovary	"	
15	Leukocyte	"	
16	muscle	"	
17			
18			
19			
20			

NOTE:

#3 gel







9/18/95 Design oligos for SOD4 to determine which ATG is the real ATG

3 oligos AAATTAACCCTCACTAAAGGGCCATCATGGCAGCGGCCGG

T3 - SOD ATG3

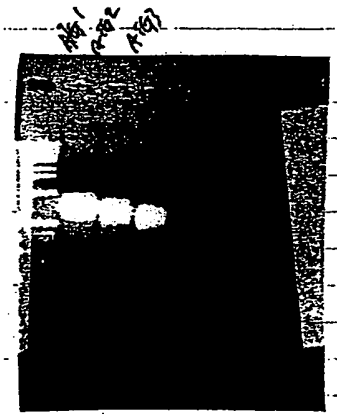
SODATG2 ATG GTCTTGGTGCAC

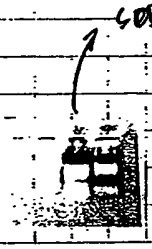
SODATG1 ATGACCTGTCAGAGC

PCR to generate pcr product for TNT

3' oligo SODEND

CGTCTAGAGGTCCTGCTCAAAGGTGGG





9/19 Subtraction Result

Sequence of two New HPT Subtracted library Come back

in HPTY which is long hyb (O/N at 42°C)

GH reduced to 10% prolactin to 0%

HPTX — short hyb is not as good

GH → 15% prolactin 3%

→ try hybridization with 5% PEG

9/20/95 TNT promega kit

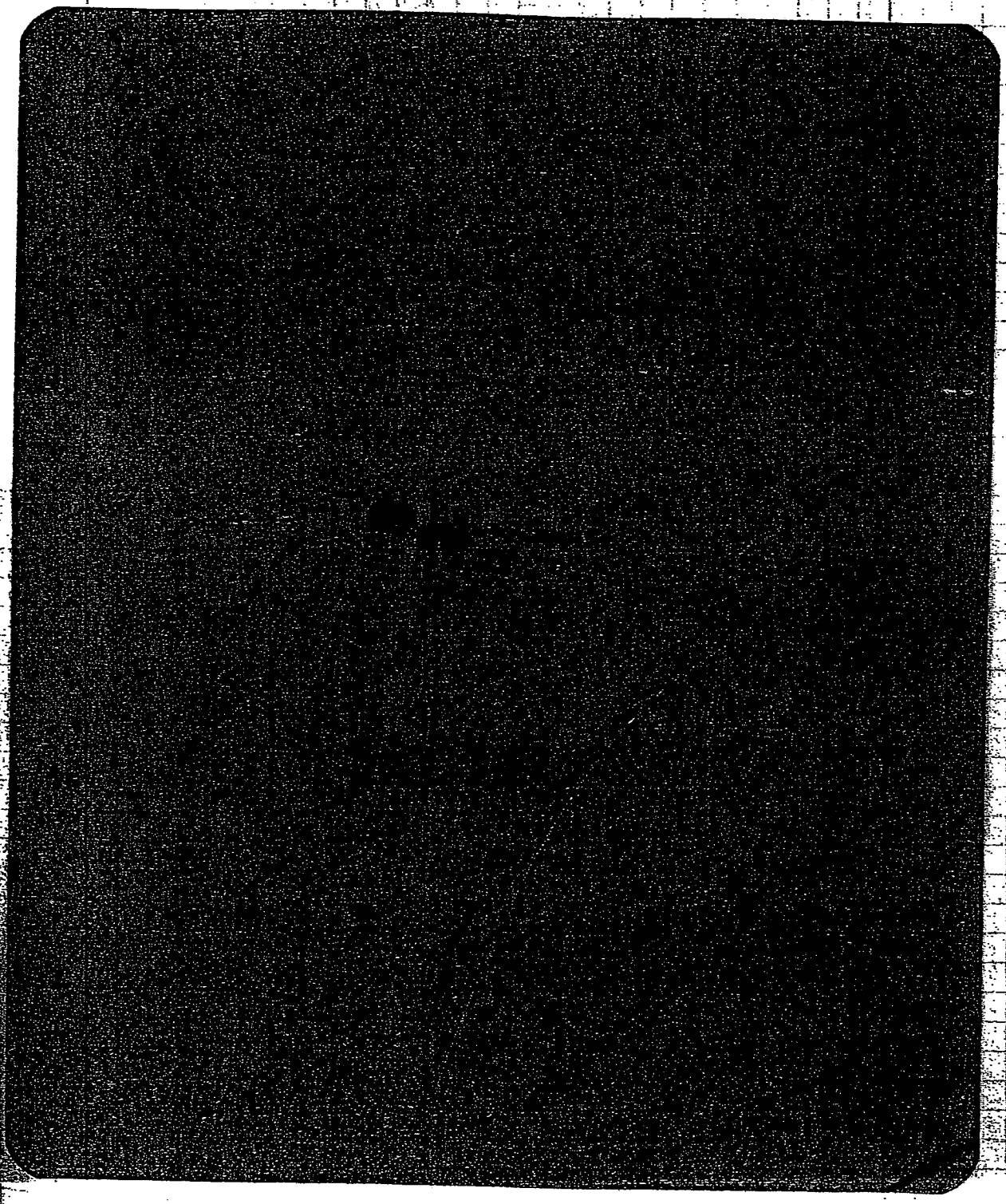
DNA: pBluescpt sk⁻
SOD full length in pBluescpt
PCR product from ΔTG1
ΔTG2
ΔTG3

T7 - CsG16 in pA2

make a premix
- 12.5 ml Rabbit Reticulocyte lysate
- 0.1 ml bf
- 0.5 ml T3 or T7 RNA polymerase
- 0.5 ml Amino Acid - met
- 2 ml 35S-met
- 0.5 λ RNase inhibitor from Boehringer
- 3 λ DNA
- 8 λ ddH2O

30°C 1hr

k



9/25 repeat TNT

10 λ reaction

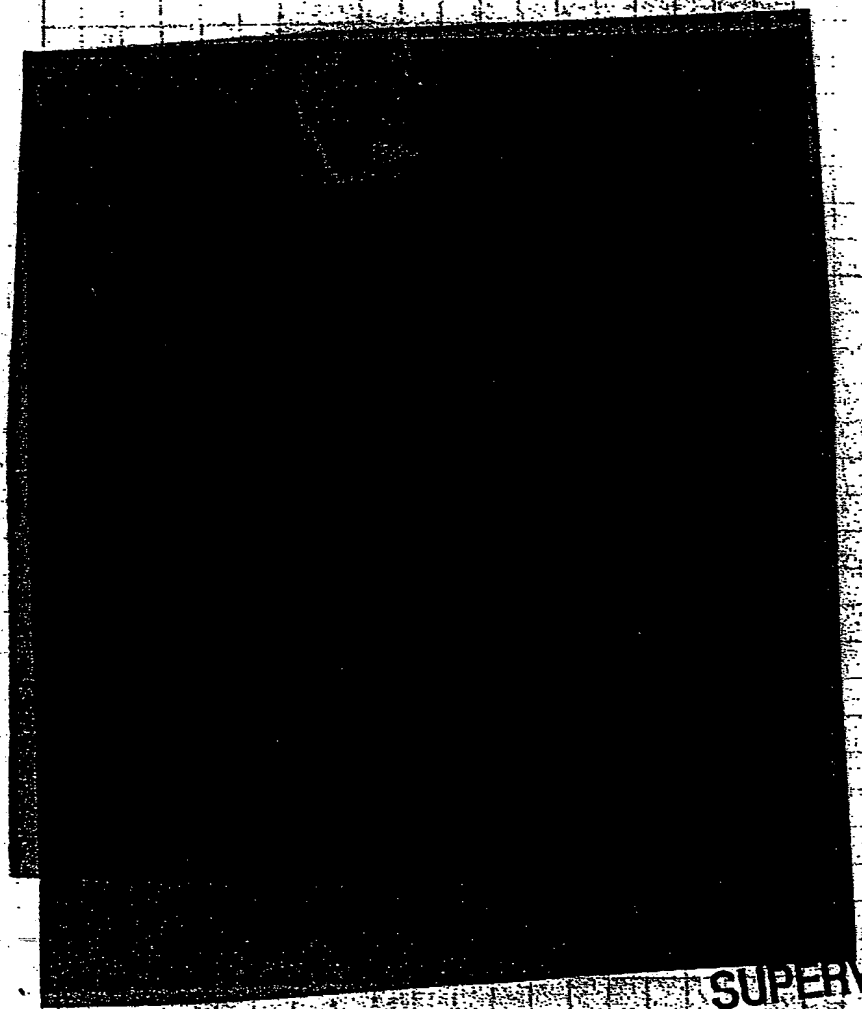
2 nl PCR product similar amount

30°C 1 hr

add 10λ loading dye boil for 2'

Spin Load 10λ on 10% SDS gel

Full length SOD1	—	0	27 kd
ATG1	—	1	24 kd
ATG2	—	2	21 kd
ATG3	—	3	



This experiment indicates there is another ATG upstream maybe the real ATG

Check seq - there is an ATG 19 AA upstream this will make the protein 2 kd

SUPERVISOR

DATE- 09/27/95

10/4/95

Subtraction result come back with a nice improvement by add 5% PEG (8000) in hybridization mix. it may be increase hybridization efficiency

result:		GH	PP
	before subtraction :	27%	7%
	after subtraction :	5%	1%

— clone Full length TMFepsilon by PCR HPD l. brain

HPD was mRNA extracted and used to PCR using

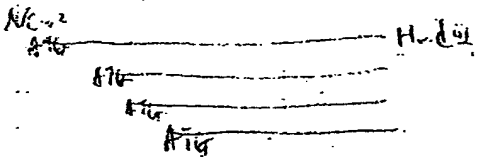
TM13 Reverse + HLTBT71Fp06
+ HLTBT71Fp04

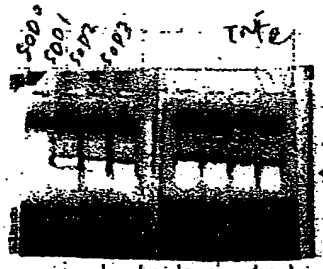
one band of 0.8 Kb seen

— Second PCR

1λ first PCR T3 + HLTBT71Fp05
Fp04

— PCR to construct SuTM4 expression vectors





- Lily will clone into 70E60

10/5/95 Gene trapper

genes: TNFR p55
TNF5
TGF β — WWH
Rad 16 — YFW

Label oligos: w/ TDT

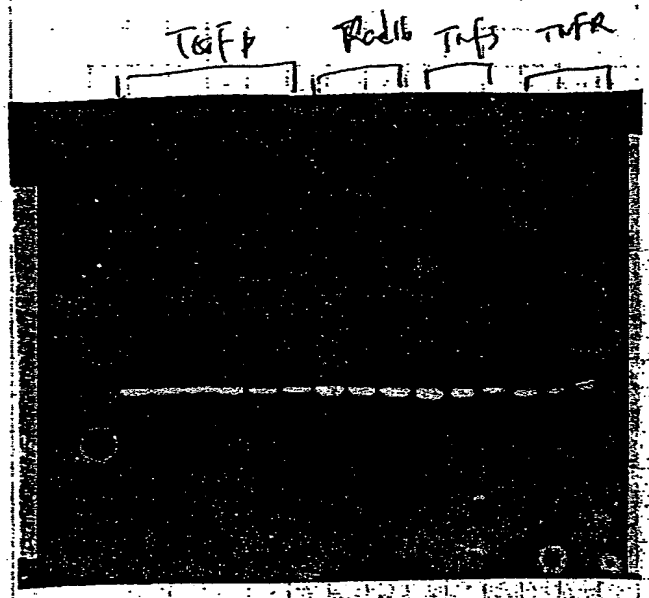
TGF β 11362
11356

TNF-5 TNF5 Rp01

TNFR p55 cap1 + Rp-2

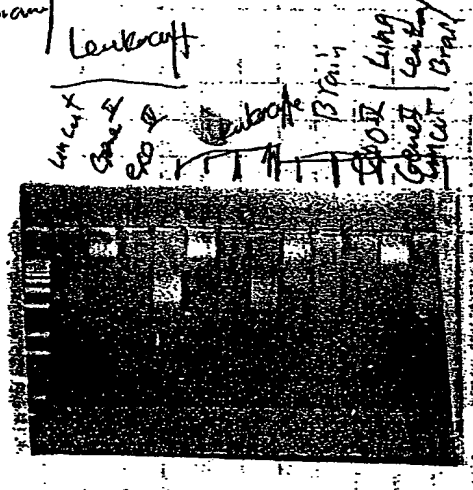
Rad16 - R8 + R11

Cabling nd working



produce SS DNA by gene T PAIN

Invitrogen library



TNF5 → Leuk

TGFβ → M

Rad 16 → L

TNFR p55 →

10/6/95 Repeat biotin labeling of oligos

ethanol ppt oligos final concentration 200ng/l

3μg DNA (12 μl)
10 λ 5x TdT bfr
5 λ biotin-ddUTP
8 λ TdT

working

10/9/95 Capture

add 6 λ 4x bfr — 95°C 1'
add 5 λ TNFR oligo + Brain
0.5 λ other oligos + Leukocyte — TNFS
Leukocyte — Rad 6
Brain — TGFβ

Hyb:

37°C 1 hr

treat SA - Magnetic beads, capture, wash

Repair all 10 λ

10/9/95

transfor to DH10B from BRL

plate	10λ	100λ
TNFRp55	—	40 colony
TNF5	—	41
TGFβ	—	1
Rad 1b	—	6

add 1ml 2x Freeze liqu to remaining

10/10/95 pCR to identify positive clones.

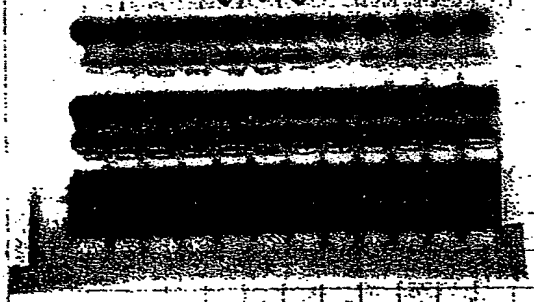
2 gene specific primers

TNF5

50% clones are positive

2 gene specific primers

TNFRp55



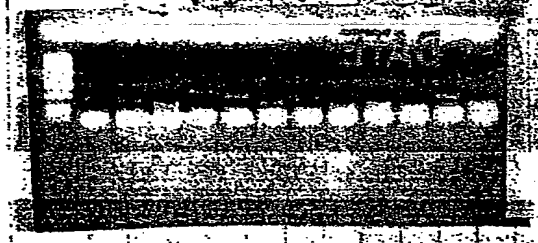
~5% clone positive

Not so colony hybr

Show only 32/100

hybridize

Sap + TNF5 Fp1



10/16/95 Fusion construct for TNFr with IL6 signal

PCR:

Oligos: IL6 BamH1

CGC GGA TCC ATG AAC CAT GAA CTC CTT CTC CAC
Met

IL6-TNFr1 IL6 TNFr

GTG GCT TCC CTG GCC GGT TGT GAG GAC AAA CTC

IL6-TNFr2

IL6 BamH1

IL6

IL6-TNFr2

IL6 TNFr1

TNFrBamH1

50 µl reaction 58 program

Fusion for Full length TNFr (HPDD012)

PCR from HpD library

M13 Rev ← HpDD012F5

T3 ← F4

R3 ← T7

SUPERVISOR- [signature]

DATE- 10/17/95

10/23 Seq analysis of 4 HTTBN61 clones

HTTBN61 S08 — wrong clone
HTTBN61 S23 — wrong clone
HTTBN61 S02 — wrong clone
HTTBN60 S07 — wrong clone

design new primers for oligo capture ?

— how Lily do a colony hybridization

— the construct of IL6/TNFr is missing kozak sequence

new oligo is made IL6's'

GCG GG ATCC GCCACC ATG TCCTTC TCCAC
BamHI kozak MET

PCR to generate IL6/TNFr fusion.

Clones are made into the two CHO vectors by Lily

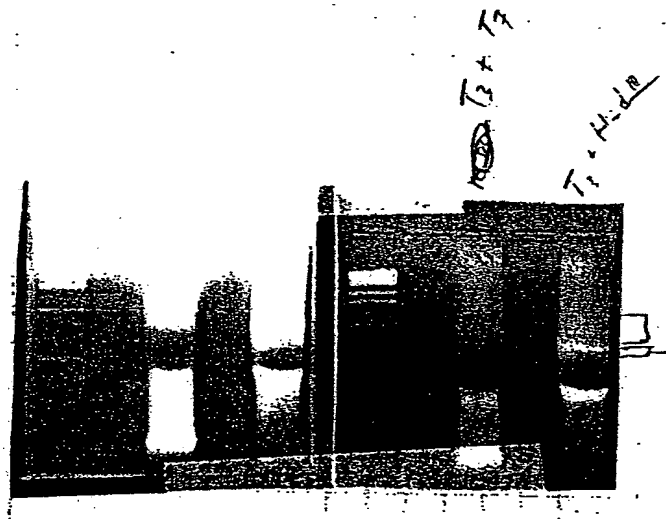
12/26 ct'l having problem get HPDD012 PCR fragment

PCR products

$\overrightarrow{M13}$ $\overrightarrow{T_3}$ +

not digested HPDD012

T_3 ← T_7 TMFSHindIII



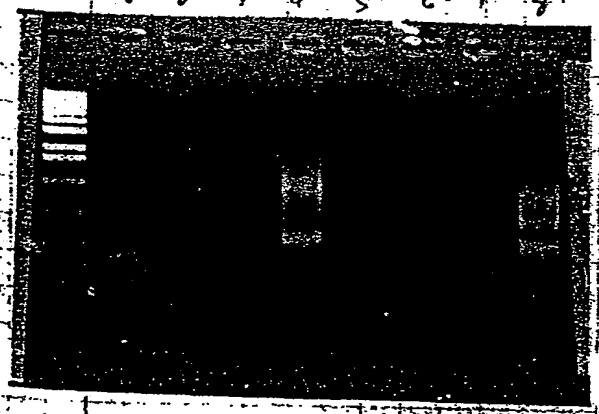
Low melting gel
Φ 2x
$CHCl_3$

digest w/ HindIII + BamHI

Clone it pbluescript HindIII + BamHI

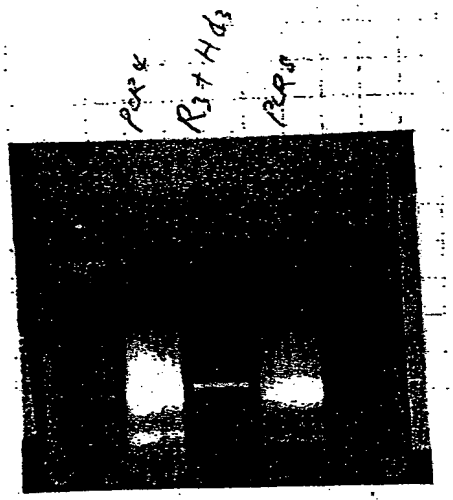
10/06

PCR products 5μl/50



	primer	template	
1	T_3+F_2		HPDD012
2	T_7+R_3		
3	TMFSHind+R3		
4	T_3+F_2	— PCR① —	HPD mutant
5	T_3+F_2		HPD
6	T_7+R_3		
7	TMFSHind+R3		
8	T_3+F_2	— PCR② —	HPD mutant

10/17 Low melting gel



Cut out DNA

PCR 4 → 0.6 - 1.1 Kb

PCR 8 - -

R3+Hd3 →
(PCR 7)

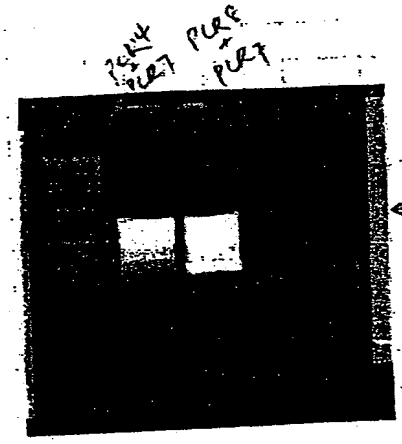
Φ 2x

CutSmart 1x

ethanol ppt

mix PCR 4 + PCR 7
PCR 8 + PCR 7

PCR using T3 + Hd3



← low melting gel purify top band

HindIII + BamHI digestion

Clone into pBluescript HindIII + BamHI digestion

PCR check → no clone found

11/7/95
oligo flanking TNFδ Fpo7 + Rpo7

PCR	DNA	primer	
①	HPD 1. brary	Rpo7 + Fpo7	
②	HPD 1. brary	Rpo7 + Fpo7 + TNFδ Bam + TNFδ Hind III	
③	HPD 1. brary	TNFδ BamHI + Hind III	
④	HLTBT71S09	Rpo7 + Fpo7	expect → 1.7 kb
⑤	HLTBT71S09	5 BamH + 3 Hind III	expect 600 bp

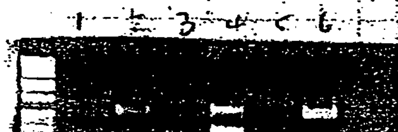
1 2 3 4 5

wrong size for δ Bam product

11/10 New primer made for TNFδ BamHI

PCR	DNA	primer
1)	HPD 1. brary	Rpo7 + Fpo7
2)	HPD 1. brary	5 BamHI + Hind III
3)	PCR ①	Rpo7 + Fpo7
④	PCR ①	BamH I + Hind III
5)	HLTBT71S09	Rpo7 + Fpo7
6)	HLTBT71S09	5 Bam I Hind III

1 2 3 4 5 6



11/11/95 2% low melting gel purification PCR 2 & PCR C

Φ/ Φ/CHCl3 extr ethanol ppt

digest with BamHI + HindIII

also digest Vector pQE9

Ligation o/n

Design oligos to test ligation for SAGE

Ligation oligo 1 GAGTCAGTTCAATGCCAAACGGCATG

Ligation oligo 2 CCGTTTGGCATTGAACTGACTCCATC

SUPERVISOR- [signature]

DATE- 11-20-95

11/20/95 RACE for HTTBN61 TNFR p55 homolog

The following library has the gene based on PCR using 2 gene specific primers

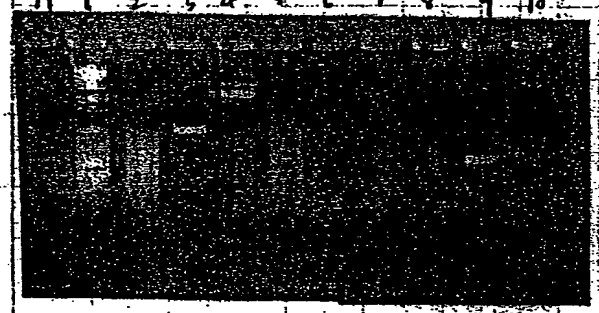
HCQ HLQ HFV HFB HPD HAP HTX HE9
1 2 3 4 5 6 7 8

MisRv T3

F3 F4

PCR using MisRv + F4

HPD
9 = TNFeRpo8 + Fpo7
10 : Rpo8 + Fpo6
11 : Rpo8 + δHindIII



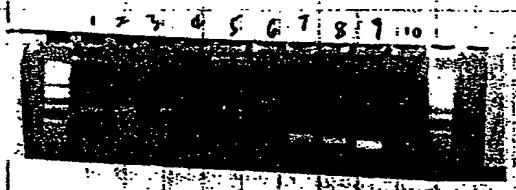
Second PCR

T3 + F3

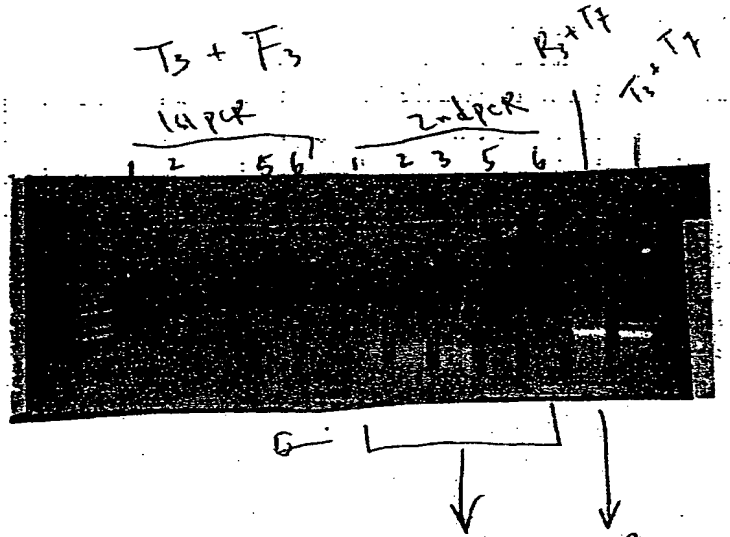
1 – 8 template PCR 1-8

9 HTTBN61

10 HTTBN61 T7 + Rpo1



11/21/95



5λ + 10% (gel purified)

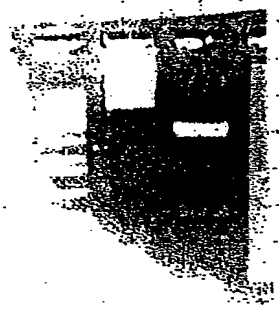
11/28

T3 + T7 PCR

no specific products

11/28 TNFδ His tag construct

Seq verified digestion w/ BamH + Hind 3



Signed by RG and SR

gave DNA to protein engineers

12/6/95 prepare linkers for SAGE

large scale oligo synthesis each 7.5 mg

linker A

TTTTAATTAACCCTCACTAAAGGGCTCGCACGGATGCATG 4368
TTAATTGGGAGTGATTTCCCGAGCGTGCCTAC 4370

linker B

TTTTGTAATACGACTCACTATAGGGCAAGTCGGATGCATG 4369
CATTATGCTGAGTGATATCCCGTTCAGCCTAC 4371

— 12% urea acrylamide gel purify the oligos
put through C18 column

— or ϕ/CHCl3 ext ethanol ppt + equal V — 1M Tris pH 7.5
3 V 60% EtOH

Concentration

Sample ID	abs 260.0 nm	abs 280.0 nm		260.0 nm / 280.0 nm	280.0 nm / 260.0 nm	
4368	0.3014	0.1906	4.5 ml/l	1.5797	0.6330	ϕ extracted
2 69	0.3548	0.2061	5.3	1.7045	0.5867	
3 70	0.3056	0.1975	4.5	1.5468	0.6465	
4 71	0.3139	0.2102	4.8	1.5171	0.6591	
5 68	0.0762	0.0489	1.1	1.5581	0.6414	gel purified
6 69	0.0768	0.0456	1.2	1.7304	0.5778	
7 70	0.1143	0.0757	1.7	1.5114	0.6616	
8 71	0.1009	0.0660	1.5	1.5292	0.6539	
9						

— Anneal
80 µg (4368) + 64 µg (4370) + 20 λ 10x Kinase buffer
80 µg (4369) + 64 µg (4371) 70 mM Tris Cl
10 mM MgCl
1 mM DTT

add H2O to 200 λ

A B gel purified

A' B' ϕ/cuts / ethanol ppt

65°C → cool to 42°C put heat block on bench

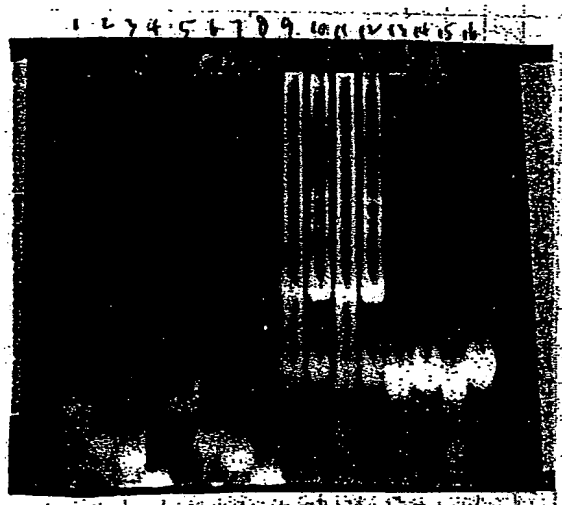
add 1λ 100 mM ATP
1λ 100 mM ddATP
2λ PNK
2λ Klenow

37°C 1 hr

take 2λ each ligate in 10λ 2λ 5× BRL ligation buf
1λ NEB high conc ligase

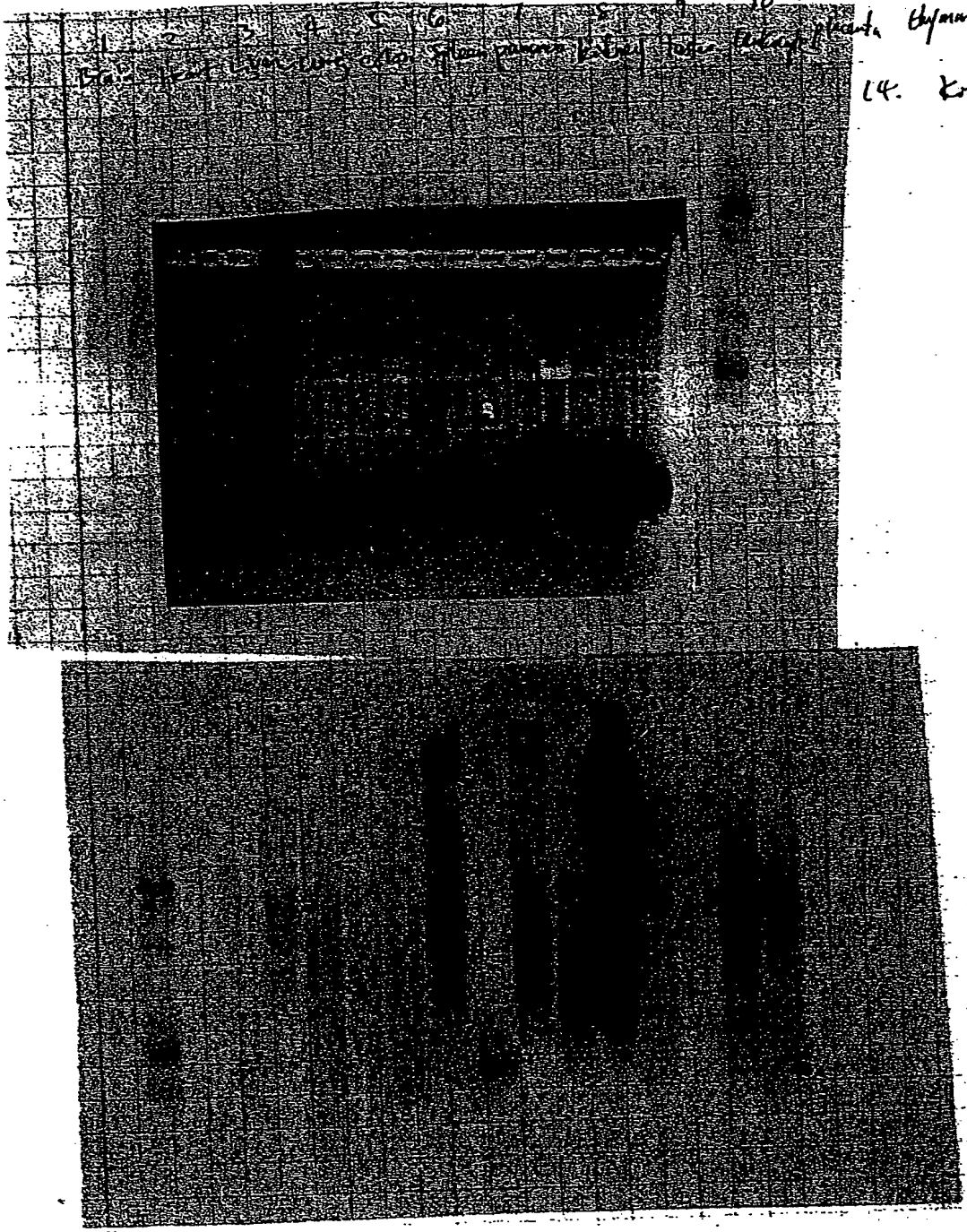
RT 2-3 hrs

12% native acrylamide gel



1	4368	ϕ/cuts ethanol ppt
2	4370	
3	4369	
4	4371	
5	4368	gel purified
6	4370	
7	4369	
8	4371	
9	Linker A	self ligation
10	Linker A'	
11	B	
12	B'	
13	Linker A	
14	A'	
15	B	
16	B'	

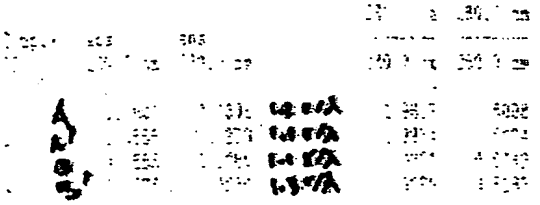
12/12/95 Northern fox HTTBM64 TMTR



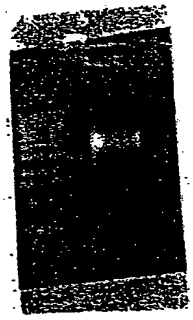
12/11/95 CAGE

Linkers A and linkers B

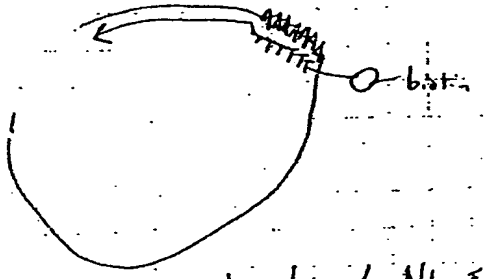
13
Gallbladder



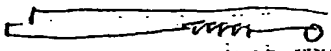
HBC library plasmid DNA digest w/ XhoI
total 7 µg



take ½ (3 µg)
↓ 90°C 5 min
add 0.5 µg biotinylated oligo dT LINKER
↓ 37°C
Klenow 1X 100 mM dNTP
30 min



↓ digest w/ NlaIII



↓ ligation in 20A PKL buffer
RT 3 hr

Linker A
0.2 µg

Linker B
1.2 µg

12/12 pre-wash ... streptavidin magnetic beads

TE wash 3X

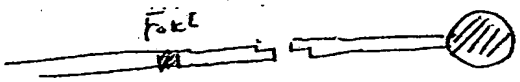
Resuspend in 30 λ TE add ligation

bind 1 hr at RT mix occasionally

wash w/ TE/1m NaCl 2X transfer to new tube

wash 2X more

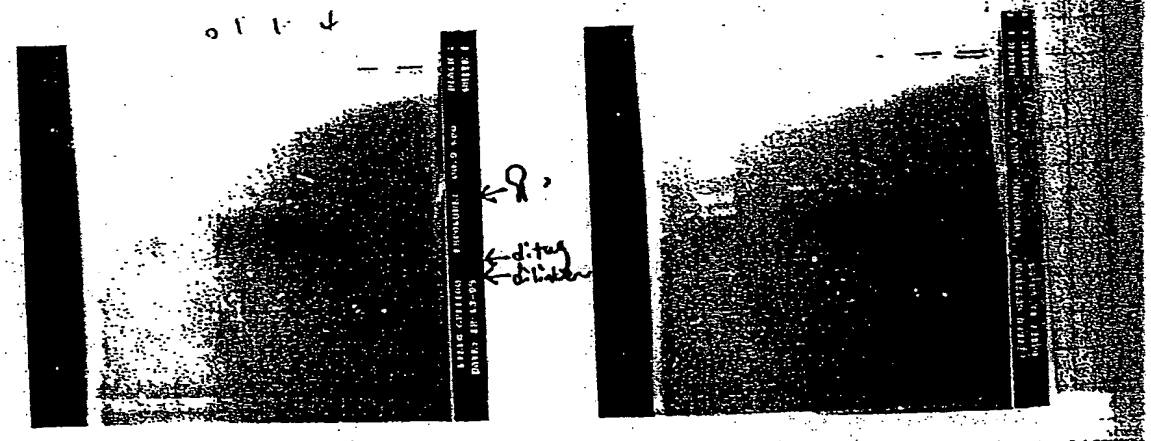
digestion with FokI at 37°C



Φ/Lots adapter ↓ T4 DNA poly nuclease
+
1λ 100mM dNTP
RT 30'

↓ Ligation
8°C
mix A + B o/N

↓ PCR T3 + T7 66 program
0.1 1. 4λ ligation as template (30 cycles)



12/12 prewash strepavidin magnetic beads

TE wash 3X

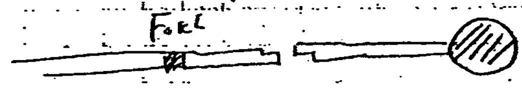
resuspend in 30λ TG add ligation

Bind 1 hr at RT mix occasionally

wash w/ TE/1M NaCl 2X, transfer to new tube

wash 2X more

digestion with FokI at 37°C



Φ/CHCl3 ethanol ppt ↓ T4 DNA polymerase + 1λ 100mM dATP

RT 30'

↓ Ligation 8°C o/n

mix A + B

↓ PCR T3 + T7 66 program (30 cycles)

0.1 1, 4λ ligation as template

0.1 1 4